

Glycogenin, the primer of glycogen synthesis, binds to actin

Susanna Baqué, Joan J. Guinovart, Juan C. Ferrer*

Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Martí i Franquès 1, E-08028 Barcelona, Spain

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Abstract We have studied the intracellular localization of glycogenin by fusing green fluorescent protein (GFP) to the N-terminus of rabbit muscle glycogenin and expressing the chimeric protein in C₂C₁₂, COS-1 and rat hepatic cells. The fusion protein showed a nuclear and cytosolic distribution and partially co-localized with actin in the cytosol. Disruption of the actin cytoskeleton with cytochalasin D led to a change in the pattern of green fluorescence, which coincided with that observed for the remaining non-depolymerized actin. The distribution of the single point mutant K324A was completely uniform and was not affected by this drug. These findings indicate that rabbit muscle glycogenin binds to actin through the heptapeptide ³²¹DNIKKKL³²⁷, a common motif found in other actin-binding proteins, which is located at the C-terminal end of this protein, and suggest that the actin cytoskeleton plays an important role in glycogen metabolism.

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Key words: Glycogenin; Glycogen metabolism; Actin cytoskeleton; Green fluorescent protein

1. Introduction

Glycogenin is an autocatalytic self-glucosylating protein involved in the de novo synthesis of glycogen (for reviews see [1,2]). This glucosyltransferase is capable of transferring a glucosyl residue from UDP-glucose to the hydroxyl group of its own tyrosine 194, and adding the subsequent 1,4-linked glucose units to form a linear oligosaccharide up to 11 glucosyl residues long [3]. Glucosylated glycogenin acts as the primer for the biogenesis of glycogen in muscle and in other tissues, and serves as the substrate for glycogen synthase [1,2].

Glycogenin was first isolated from rabbit skeletal muscle [4,5] but, in contrast to what occurs for glycogen synthase, it does not appear to present isoforms characteristic of different tissues [6–8]. Nevertheless, its relative abundance varies greatly from one cell type to another. By Northern blot analysis, glycogenin mRNA has been shown to be most abundant in skeletal and cardiac muscle, also present in lung, brain, kidney and testis but barely detectable in liver [7,8]. At the protein level, glycogenin is present in a molar ratio of 1:1 with glycogen synthase in rabbit skeletal muscle [9], whereas in rabbit liver its abundance is 200-fold lower [6].

Recently, the subcellular distribution of glycogenin in different tissues has been examined by histochemical techniques. In neuronal cells of chicken retina, endogenous glycogenin is present in both the cytoplasm and the cell nucleus [10]. In contrast, in rat 1 fibroblasts stably overexpressing rabbit

muscle glycogenin, the enzyme was only detected diffusely in the cytoplasm, and its distribution was found not to be affected by the presence or absence of glucose in the medium [11].

We have recently shown that in isolated rat hepatocytes, glycogen synthase translocates, in response to glucose, from a uniform cytosolic distribution to the cell cortex. In this movement towards the cell periphery, a marked increase in the co-localization of the protein with actin filaments was observed [12].

Here, we use the intrinsic fluorescence of the *Aequoria victoria* green fluorescent protein (GFP) [13] to study the subcellular distribution of glycogenin. We constructed a chimeric protein by fusing GFP to the amino-terminal end of rabbit muscle glycogenin (GFP/glycogenin), and the localization of the fusion protein overexpressed in three different types of cells (C₂C₁₂, rat hepatocytes and COS-1) was analyzed by confocal microscopy.

The results obtained with this procedure show that glycogenin is mainly found in the cytoplasm, partly bound to actin, with a minor fraction in the nucleus. This distribution is not affected by the presence or absence of glucose in the incubation medium, but it is altered by disrupting the actin cytoskeleton with cytochalasin D.

2. Materials and methods

2.1. Plasmid construction

Standard molecular cloning techniques were used throughout [14]. Total RNA, isolated from rabbit skeletal muscle using the RNagents total RNA isolation system (Promega), was used to prepare cDNA with the RT-PCR kit (Stratagene). Glycogenin cDNA was amplified following the supplier's instructions and using the sense primer (Boehringer Mannheim) GACCATATGACAGATCAGGCCTTTGTGACACTGACC, which contains a *Nde*I site at the start codon (underlined) and 10 codons complementary to amino acids 1–10 of glycogenin, and the antisense primer CCCAGCTCTAGATGCTAAGTATCAGAAACAAGTGCC, which contains a *Xba*I site (underlined) and is complementary to nucleotides 1098–1133 of the 3' untranslated region of the rabbit muscle glycogenin cDNA (GenBank accession number L01791) [7]. The PCR product was ligated into the *Sma*I site of pUC18 using the SureClone ligation kit (Pharmacia Biotech). The resulting pUC18/glycogenin vector was digested with *Nde*I and blunt-ended with the Klenow fragment of *Escherichia coli* DNA polymerase I and dNTPs (Promega), followed by digestion with *Sal*I. The fragment containing the full coding sequence of rabbit muscle glycogenin was ligated into pEGFP-C1 (Clontech) which had been previously digested with *Bgl*II, Klenow-filled, and digested with *Sal*I. This ensured the in-frame fusion of glycogenin at the C-terminus of the GFP coding sequence plus a linker of 5 amino acids, under the control of the constitutive immediate early promoter of the human cytomegalovirus.

The K324A mutation in the sequence of glycogenin was introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and the two oligonucleotides CCTTTGACAACATCGCGAAGAACTTGACAC (sense) and GTGTCAAGTTTCTTCGCGATGTTGTCAAAGG (antisense), which introduced a *Nru*I site for diagnostic purposes. The final plasmids pEGFP-C1/glycogenin and pEGFP-C1/

*Corresponding author. Fax: (34) (3) 4021219.
E-mail: ferrer@sun.bq.ub.es

Abbreviations: GFP, green fluorescent protein

Fig. 1. Expression of GFP/glycogenin in three different cell types. Confocal images of GFP/glycogenin in C₂C₁₂ cells (A), primary cultured hepatocytes (D) and COS-1 cells (G). The same population of each cell type labelled with Texas red-conjugated phalloidin: C₂C₁₂ cells (B), hepatocytes (E) and COS-1 cells (H). Co-localization pixels (where green and red overlap) are shown in white (C, F, I). Scale bar = 10 μ m.

K324A-glycogenin were purified by ionic exchange chromatography (Qiagen) and finally dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. The DNAs encoding the fusion proteins were sequenced in their entirety to confirm the absence of spurious mutations, using the ABI-PRISM DNA sequencing kit and the ABI-PRISM 377 automatic DNA sequencer (Perkin Elmer Applied Biosystems).

2.2. Cell culture and transfection

C₂C₁₂ myoblasts (ATCC CRL-1772) were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM; Whittaker), supplemented with 25 mM glucose, 10% fetal bovine serum (FBS; Biological Industries) and penicillin/streptomycin (Boehringer Mannheim). Differentiation into myotubes was induced by incubating the cells in DMEM containing 10% horse serum (HS; Life Technologies) instead of FBS. Fused myotubes were observed 3–4 days after the medium change [15]. COS-1 cells (ATCC CRL-1650) were grown in DMEM supplemented with 25 mM glucose, 10% FBS and penicillin/streptomycin. Liposome-mediated transfection of C₂C₁₂ and COS-1 cells was performed at 70–80% confluence, using 4 μ g of Clontech (Clontech) and 4 μ g of plasmid DNA per 35 mm dish, following the manufacturer's instructions. After transfection (4–5 h) at 37°C in humidified 5% CO₂:95% air, cells were washed in phosphate-buffered saline (PBS) and C₂C₁₂ cells were incubated in DMEM supplemented with 25 mM glucose and 10% HS. COS-1 cells were incubated in DMEM containing 25 mM glucose and 10% FBS. Prior to the experiments, performed 48 h after transfection, both cell types were preincubated for 4–5 h in plain DMEM without glucose.

Hepatocytes were isolated from 24 h starved male Wistar rats (Interfauna) by collagenase perfusion as described [16]. Cells were resuspended in DMEM supplemented with 10 mM glucose, 10% FBS, 100 nM insulin (Sigma), 100 nM dexamethasone (Sigma), and penicillin/streptomycin and seeded (6×10^4 cells/cm²) onto gelatin-coated glass coverslips placed on tissue culture plates. After 4 h at 37°C in humidified 5% CO₂:95% air, unattached cells were removed by washing with PBS, and transfection was performed as described above. After transfection, cells were washed in PBS and incubated in plain DMEM without glucose for 24 h. On the day of the experiment the three cell types were incubated in plain DMEM without glucose (control) or with 25 mM glucose and/or 10 nM insulin, for 3 h.

To disrupt the actin cytoskeleton, cells were incubated for 2 h in medium containing cytochalasin D (Sigma), at a concentration of 1 μ M for C₂C₁₂ and COS-1 cells and 25 μ M for hepatocytes. At the end of the incubations, cells were washed twice with PBS, fixed for 20 min in PBS containing 4% paraformaldehyde and were washed several times with PBS. For actin filament staining, fixed cells were incubated for 5 min with cold acetone (–20°C), washed extensively with PBS, and finally incubated at room temperature for 20 min with Texas red-conjugated phalloidin (Molecular Probes Europe), diluted 1:50 in PBS. Coverslips were air-dried and finally mounted onto glass slides, using the Immuno Fluore mounting medium (ICN Biomedicals, Inc.).

2.3. Confocal microscopy

Fluorescence images were obtained with a Leica TCS 4D (Leica Lasertechnik, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope and 63 \times and 100 \times (NA 1.4 oil) Leitz Plan-Apo objectives. The light source was an argon/krypton laser (75 mW). Green fluorescence (from GFP recombinants) and Texas red (used as a marker for actin filaments) were excited simultaneously at 488 and 568 nm respectively with the laser. Optical sections (0.1 μ m) were obtained and co-localization analysis was performed with Multicolor software (version 2.0, Leica

Lasertechnik) as described [17]. The confocal co-localization, defined as the topographical overlap of fluorescent markers (GFP (green), and Texas red) for the two cellular components, was represented in a cytofluorogram, in which the area where the markers overlap was indicated in yellow. By image treatment using the confocal system to illustrate the sites at which the two proteins co-localized, new images were generated in which co-localization is indicated in white.

3. Results

3.1. Expression of GFP/glycogenin in muscle cells

A proportion of C₂C₁₂ cells (5–10%) transfected with the pEGFP-C1/glycogenin construct and incubated in a glucose-free medium appeared, 2 days after transfection, strongly fluorescent. The label was found mainly in the cytosol and also in the cell nucleus (Fig. 1A), and no change was observed in the subcellular distribution of the chimeric GFP/glycogenin protein in the cells incubated with glucose and/or insulin (data not shown). Surprisingly, while the control cells transfected with the pEGFP-C1 plasmid, encoding GFP, presented uniform diffuse cytosolic and nuclear staining (data not shown), the cytosolic fraction of the GFP/glycogenin chimera exhibited a mesh-like pattern. When cultured muscle cells were stained with Texas red-conjugated phalloidin (Fig. 1B), a portion of the green fluorescence from the GFP/glycogenin fusion protein coincided with actin staining in the same area of the cytosol (Fig. 1C).

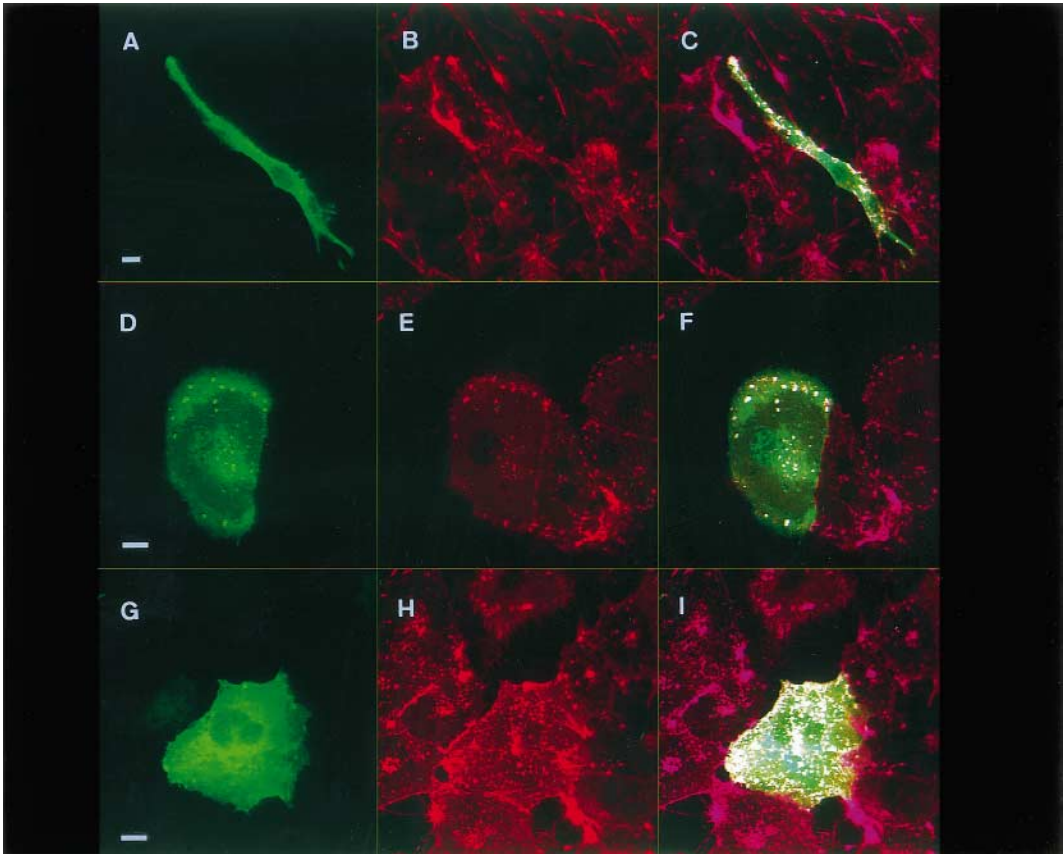
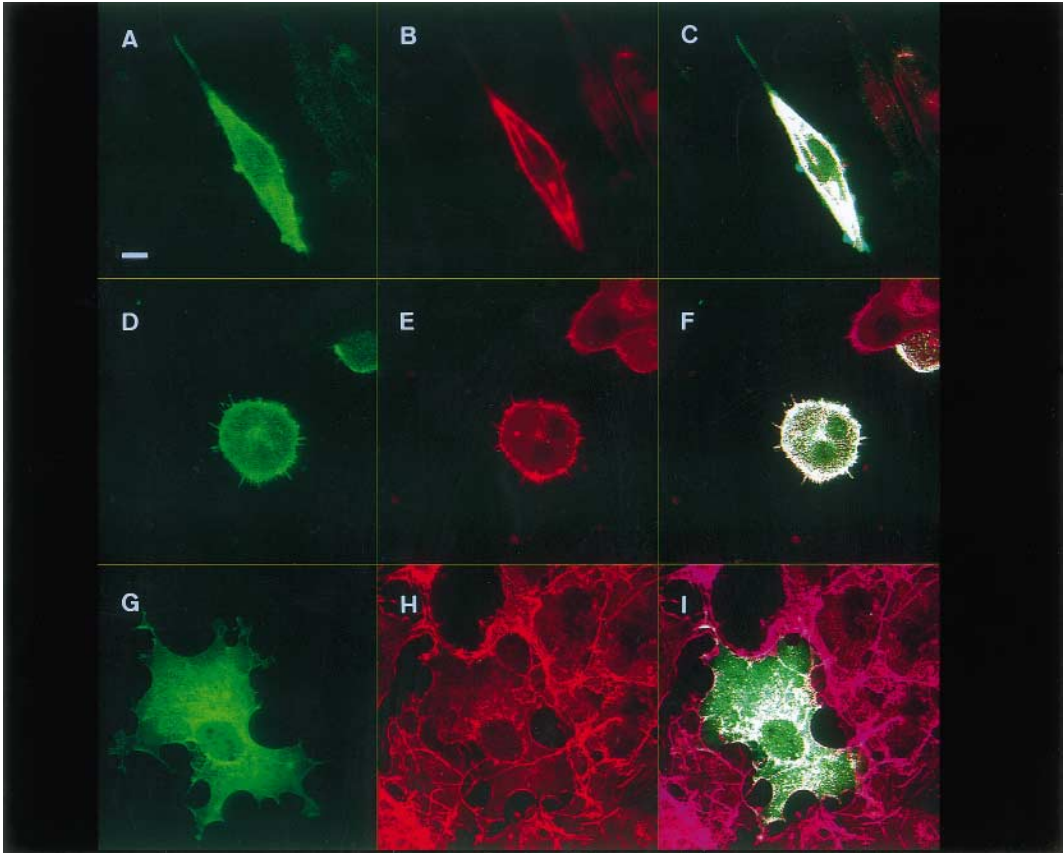
3.2. Expression of GFP/glycogenin in rat hepatocytes and COS-1 cells

In order to ascertain whether the observed distribution of glycogenin was characteristic of muscle cells or an intrinsic property of the protein, we expressed the GFP/glycogenin chimera in hepatocytes and COS-1 cells. The efficiency of transfection was higher in COS-1 cells (40–50%) than in rat hepatocytes (5–10%), but in both cases the transfected cells exhibited fluorescent labeling in the cytoplasm and the cell nucleus (Fig. 1D,G). As with C₂C₁₂, the subcellular localization of glycogenin did not change upon incubation with glucose and/or insulin (data not shown). Again, staining with Texas red-conjugated phalloidin indicated that part of the cytosolic green label co-localized with red stained actin (Fig. 1E,F,H,I).

3.3. Experiments with cytochalasin D

The treatment of the different cell types used in this study with cytochalasin D confirmed the interaction between glycogenin and actin in the cytoplasm. While the diffuse uniform pattern of fluorescence observed in cells transfected with the pEGFP-C1 plasmid was not modified by incubation with this drug (data not shown), pEGFP-C1/glycogenin-transfected

Fig. 2. Expression of GFP/glycogenin in cells incubated for 2 h in a medium containing cytochalasin D. Confocal images of GFP/glycogenin in C₂C₁₂ cells (A), primary cultured hepatocytes (D) and COS-1 cells (G). The same population of each cell type labelled with Texas red-conjugated phalloidin: C₂C₁₂ cells (B), hepatocytes (E) and COS-1 cells (H). Co-localization pixels (where green and red overlap) are shown in white (C, F, I). Scale bars = 10 μ m.



cells, when treated with cytochalasin D, showed a dramatic change in the green fluorescence pattern. In these conditions, the GFP/glycogenin chimera concentrated on the remaining non-depolymerized actin (Fig. 2).

3.4. Expression of the GFP/K324A-glycogenin variant

An analysis of the sequence of rabbit muscle glycogenin revealed the presence of the basic DNIKKKL heptapeptide at amino acids 321–327, close to the C-terminal end of the enzyme. A similar sequence (DAIKKKL) occurs in cofilin, and in other actin-binding proteins [18].

To determine whether the ³²¹DNIKKKL³²⁷ heptapeptide is responsible for the binding of the enzyme to actin, we proceeded with a loss of function approach. Lysine 324, the central residue of the motif, was replaced by an alanine and the GFP/K324A-glycogenin variant was expressed in C₂C₁₂, COS-1 cells and cultured hepatocytes. In all cases, the mutated chimeric protein showed a diffuse pattern in the cytosol and in the nucleus (data not shown), similar to that exhibited by cells transfected with the pEGFP-C1 plasmid. In contrast to what was observed for the cells expressing the wild-type glycogenin construct (GFP/glycogenin), the pattern of the mutated fusion protein (GFP/K324A-glycogenin) was not altered after incubation with cytochalasin D. This shows that the interaction between glycogenin and actin is suppressed by the single change introduced in the basic DNIKKKL heptapeptide, and strongly suggests that this fragment is part of the actin-binding site in glycogenin.

4. Discussion

We have expressed a GFP/glycogenin fusion protein in C₂C₁₂ and COS-1 cells, and in primary cultured rat hepatocytes. In all cases the chimeric fluorescent protein was readily detectable by confocal laser microscopy, and mainly localized to the cytoplasm of the cells, with a smaller fraction found in the nucleus.

While glycogenin possesses a molecular mass of 37 kDa, below the ca. 45 kDa exclusion limit for passive diffusion through the nuclear membrane porus [19], the GFP-glycogenin fusion protein has a molecular mass of 64 kDa, well above this limit. The detection of fluorescence in the nucleus of the cells transiently expressing this construct suggests that glycogenin may be actively transported into this cellular compartment. Two recent reports have described the subcellular distribution of glycogenin in two cell types. Using immunofluorescence techniques, the enzyme was found in the cytoplasm and the nucleus of chicken retina cells [10], while in rat 1 fibroblasts stably overexpressing rabbit muscle glycogenin, it was detected exclusively in the cytoplasm [11]. While we cannot explain these differences, they could be due to limitations of the detection technique or to a different behavior of this enzyme in various cell types. We have recently shown that recombinant muscular glycogen synthase, when expressed in C₂C₁₂, COS-1 and rat hepatic cells, concentrates in the nucleus under certain metabolic conditions [20]. It has also been shown that endogenous glycogen synthase and an active synthesis of glycogen both exist in the nucleus of ascites tumor cells [21,22]. Therefore, the presence of glycogenin in this cellular compartment may be physiologically relevant in certain cell types.

In contrast to what occurs with both known isoforms of

glycogen synthase, which translocate between different compartments in response to glucose [12,20], the subcellular localization of the GFP/glycogenin fusion protein was not altered by the presence or the absence of glucose and/or insulin in the medium. Interestingly, in both conditions the cytosolic fraction of the GFP/glycogenin fusion protein partly co-localized with actin. The interaction between glycogenin and actin has been confirmed through the use of cytochalasin D, an actin depolymerizing drug. The destructure of the actin cytoskeleton is accompanied by a dramatic change of the cellular distribution of the chimeric GFP/glycogenin protein in the three cell types studied. All these observations are of special interest in the case of the C₂C₁₂ cells, a cell line derived from muscle tissue. The relative abundance of glycogenin is much greater in muscle cells than in other tissues [7–9]. Native glycogenin isolated from rabbit muscle [23] and from cultured quail embryo muscle [24] is phosphorylated. This post-translational modification, which is induced by insulin [25], has been proposed to have a regulatory role [26]. We have not observed any significant differences in the cellular localization of glycogenin in cells incubated in the presence or absence of insulin, suggesting that this hormone does not have an effect on the subcellular localization of the enzyme.

Rabbit muscle glycogenin contains the basic heptapeptide DNIKKKL near the C-terminus of the protein, which is highly homologous to other sequences present in some actin-binding proteins. For instance, cofilin contains the DAIKKKL sequence [18], DSIKKKL is present in actophorin [27], DAIKKKM in tropomyosin [28], DAIKKKF in desmin [29] and in the actin depolymerizing factor [30]. Furthermore, actin binds *in vitro* to the isolated synthetic heptapeptide DAIKKKL [31]. Our results indicate that this motif in the primary sequence of glycogenin is responsible for its binding to actin. The study of the cellular distribution of a variant form of the enzyme in which lysine 324 was replaced by an alanine residue (K324A-glycogenin) confirmed that the mutated enzyme did not interact with actin. In the primary sequence of the human skeletal muscle glycogenin, a highly homologous fragment (DNIKRKL) can be found, which only presents the conservative change of the second lysine residue of the motif to an arginine, another basic residue. Although the glycogenins from the two different sources exhibit a very high degree of identity throughout the entire sequence (93%) [8], the conservation of this fragment may indicate its importance for the function of the enzyme.

More interestingly, the glucokinases characterized from rat [32], mouse [33] and human [34] tissues all contain the DAIKRR sequence, again highly homologous to that found in actin-binding proteins as well as in glycogenin. Rat glucokinase from liver and islet pancreatic β -cells, overexpressed in COS-7 cells, as well as endogenous rat liver glucokinase have been shown to co-localize with actin filaments [35]. Furthermore, overexpression of glucokinase, but not of hexokinase I, in cultured hepatocytes triggers the activation of glycogen synthase and stimulates the synthesis of glycogen [36], suggesting a close interaction between the two enzymes in a common cellular compartment. Using a similar approach to that described here, we are currently investigating the metabolic implications of the actin-binding capabilities of glucokinase.

The translocation of the hepatic glycogen synthase towards the cell cortex induced by glucose correlates with a marked increase in the co-localization with actin filaments [12]. More-

over, glycogen synthesis is potently inhibited by cytochalasins in cultured rat hepatocytes, by a undefined indirect mechanism that does not involve the covalent inactivation of glycogen synthase. A possible role for the actin cytoskeleton was suggested [37].

Taken together, all these observations are a clear indication of the important role that the actin cytoskeleton plays in the metabolism of glycogen, in a yet undefined manner. Further studies are required to elucidate the exact role of this cellular structure and how it constitutes, probably through the control of the cellular localization of the enzymes implicated, an additional regulatory mechanism of the glycogen synthesis and degradation processes.

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